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(54) Title: PROCESS FOR THE FERMENTATIVE PREPARATION OF L-AMINO ACIDS WITH AMPLIFICATION OF THE ZWF GENE

(57) Abstract: The invention relates to a process for the preparation of L-amino acids by fermentation of coryneform bacteria, which comprises carrying out the following steps: a) fermentation of the desired L-amino acid-producing bacteria in which at least the zwf gene is amplified, b) concentration of the L-amino acid in the medium or in the cells of the bacteria andc) isolation of the L-amino acid produced.

Process for the fermentative preparation of L-amino acids with amplification of the zwf gene

The invention relates to a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, using coryneform bacteria in which at least the zwf gene is amplified.

Prior art

L-Amino acids are used in animal nutrition, in human medicine and in the pharmaceuticals industry.

It is known that amino acids are prepared by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of its great importance, work is constantly being undertaken to improve the preparation process. Improvements to the process can relate to fermentation measures, such as e. g. stirring and supply of oxygen, or the composition of the nutrient media, such as e. g. the sugar concentration during the fermentation, or the working up to the product form by e. g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e. g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acids such as e. g. threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium glutamicum strains which produce L-amino acids.

Object of the invention

The inventors had the object of providing new improved processes for the fermentative preparation of L-amino acids with coryneform bacteria.

Description of the invention

L-Amino acids are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and especially in animal nutrition. There is therefore a general interest in providing new improved processes for the preparation of amino acids.

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, using coryneform bacteria in which the nucleotide sequence which codes for the Zwf protein (zwf gene) is amplified, in particular over-expressed.

The strains employed preferably already produce L-amino acids before amplification of the zwf gene.

Preferred embodiments are to be found in the claims.

The term "amplification" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of

coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among specialists for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are, for example, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants prepared therefrom,

such as, for example, the L-threonine-producing strains

Corynebacterium glutamicum ATCC21649
Brevibacterium flavum BB69
Brevibacterium flavum DSM5399
Brevibacterium lactofermentum FERM-BP 269
Brevibacterium lactofermentum TBB-10

and such as, for example, the L-isoleucine-producing strains

Corynebacterium glutamicum ATCC 14309
Corynebacterium glutamicum ATCC 14310
Corynebacterium glutamicum ATCC 14311
Corynebacterium glutamicum ATCC 15168
Corynebacterium ammoniagenes ATCC 6871

and such as, for example, the L-tryptophan-producing strains

Corynebacterium glutamicum ATCC21850 and
Corynebacterium glutamicum KY9218(pKW9901)

and such as, for example, the L-lysine-producing strains
Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum ATCC13032
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DSM12866.

It has been found that coryneform bacteria produce L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, in an improved manner after over-expression of the zwf gene which codes for the Zwf protein.

Alleles of the zwf gene which result from the degeneracy of the genetic code or due to sense mutations of neutral function can furthermore be used.

JP-A-09224661 discloses the nucleotide sequence of the glucose 6-phosphate dehydrogenase gene, called zwf, of Brevibacterium flavum MJ-223 (FERM BP-1497). JP-A-09224661 describes the N-terminal amino acid sequence of the Zwf polypeptide as Met Val Ile Phe Gly Val Thr Gly Asp Leu Ala Arg Lys Lys Leu.

However, it has not been possible to confirm this. Instead, the following N-terminal amino acid sequence has been found: Val Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp. The valyl radical in the N-position can be split off in the context of post-translational modification, and Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp is then obtained as the N-terminal amino acid sequence.

To achieve an amplification (e. g. over-expression), the number of copies of the corresponding genes is increased, or the promoter and regulation region or the ribosome

binding site upstream of the structural gene is mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-amino acid formation. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs are either present here in plasmids with a varying number of copies, or are integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification EPS 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and in known textbooks of genetics and molecular biology.

By way of example, the Zwf protein was over-expressed with the aid of a plasmid. The E. coli - C. glutamicum shuttle vector pEC-T18mob2 shown in Figure 1 was used for this. After incorporation of the zwf gene into the KpnI/SalI cleavage site of pEC-T18mob2, the plasmid pEC-T18mob2zwf shown in Figure 2 was formed.

Other plasmid vectors which are capable of replication in C. glutamicum, such as e.g. pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pZ8-1 (EP-B- 0 375 889), can be used in the same way.

In addition, it may be advantageous for the production of L-amino acids to amplify one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate pathway or of amino acid export, in addition to amplification of the zwf gene.

Thus, for example, in particular for the preparation of L-threonine, one or more genes chosen from the group consisting of:

- the hom gene which codes for homoserine dehydrogenase (Peoples et al., Molecular Microbiology 2, 63-72 (1988)) or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase (Archer et al., Gene 107, 53-59 (1991),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns et al., Journal of Bacteriology 174: 6076-6086 (1992)),
- the pyc gene which codes for pyruvate carboxylase (Peters-Wendisch et al., Microbiology 144: 915-927 (1998)),
- the mqo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the tkt gene which codes for transketolase (accession number AB023377 of the European Molecular Biologies Laboratories databank (EMBL, Heidelberg, Germany)),

- the gnd gene which codes for 6-phosphogluconate dehydrogenase (JP-A-9-224662),
- the thrE gene which codes for threonine export (DE 199 41 478.5; DSM 12840),
- the zwa1 gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE: 199 41 478.5)

can be amplified, in particular over-expressed, at the same time.

Thus, for example, in particular for the preparation of L-lysine, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the lysC gene which codes for a feed back resistant aspartate kinase (Kalinowski et al. (1990), Molecular and General Genetics 224: 317-324),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- the tkt gene which codes for transketolase (accession number AB023377 of the European Molecular Biologies Laboratories databank (EMBL, Heidelberg, Germany)), «
- the gnd gene which codes for 6-phosphogluconate dehydrogenase (JP-A-9-224662),

- the lysE gene which codes for lysine export (DE-A-195 48 222),
- the zwa1 gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE 199 47 791.4)

can be amplified, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-amino acids at the same time to attenuate one of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE 199 51 975.7; DSM 13114),
- the zwa2 gene (DE: 199 59 327.2; DSM 13113)

in addition to the amplification of the zwf gene.

In addition to over-expression of the Zwf protein, it may furthermore be advantageous for the production of L-amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of L-amino acid production. A summary of known culture methods is described in the textbook by Chmiel

(Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular microorganisms in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e. g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e. g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e. g. glycerol and ethanol, and organic acids, such as e. g. acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture. Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture. Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances

mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of L-amino acid has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51:. 1167-1174).

The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

Escherichia coli K-12 DH5α/pEC-T18mob2 as DSM 13244

The following figures are attached:

- Figure 1: Map of the plasmid pEC-T18mob2
- Figure 2: Map of the plasmid pEC-T18mob2zwf
- Figure 3: Map of the plasmid pAMC1
- Figure 4: Map of the plasmid pMC1
- Figure 5: Map of the plasmid pCR2.1poxBint

The base pair numbers stated are approx. values obtained in the context of reproducibility.

Re Figure 1 and 2:
The abbreviations used have the following meaning:

Tet:	Resistance gene for tetracycline
oriV:	Plasmid-coded replication origin of E. coli
RP4mob:	mob region for mobilizing the plasmid
rep:	Plasmid-coded replication origin from C. glutamicum plasmid pGA1
per:	Gene for controlling the number of copies from pGA1
lacZ-alpha:	lacZα gene fragment (N-terminus) of the β-galactosidase gene
lacZalpha':	5'-Terminus of the lacZα gene fragment
'lacZalpha:	3'-Terminus of the lacZα gene fragment

Re Figure 3 and 4:
The abbreviations used have the following meaning:

Neo r :	Neomycin/kanamycin resistance
ColE1 ori:	Replication origin of the plasmid ColE1
CMV:	Cytomegalovirus promoter
lacP:	Lactose promoter
pgi:	Phosphoglucose isomerase gene
lacZ:	Part of the β-galactosidase gene
SV40 3' splice	3' splice site of Simian virus 40

SV40 polyA:	Polyadenylation site of Simian virus 40
f1(-)ori:	Replication origin of the filamentous phage f1
SV40 ori:	Replication origin of Simian virus 40
kan r:	Kanamycin resistance
pgi insert:	Internal fragment of the pgi gene
ori:	Replication origin of the plasmid pBGS8

Re Figure 5:
The abbreviations used have the following meaning:

ColE1 ori:	Replication origin of the plasmid ColE1
lacZ:	Cloning relict of the lacZα gene fragment
f1 ori:	Replication origin of phage f1
KmR:	Kanamycin resistance
ApR:	Ampicillin resistance
poxBint:	Internal fragment of the poxB gene

The meaning of the abbreviations for the various restriction enzymes (e. g. BamHI, EcoRI etc.)are known from the prior art and are summarized, for example, by Kessler and Höltke (Gene 47, 1-153 (1986)) or Roberts et al. (Nucleic Acids Research 27, 312-313 (1999)).

Examples

The following examples will further illustrate this invention. The molecular biology techniques, e.g. plasmid DNA isolation, restriction enzyme treatment, ligations, standard transformations of Escherichia coli etc. used are, (unless stated otherwise), described by Sambrook et al., (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratories, USA).

Example 1

Expression of the Zwf protein

1.1 Preparation of the plasmid pEC-T18mob2

The E. coli - C. glutamicum shuttle vector pEC-T18mob2 was constructed according to the prior art. The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting tetA(Z) gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number AF121000), the replication region oriV of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the lacZα gene fragment including the lac promoter and a multiple cloning site (mcs) (Norrander et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al.,(1983) Bio/Technology 1:784-791). The vector constructed was transformed in the E. coli strain DH5α (Brown (ed.) Molecular Biology Labfax, BIOS Scientific Publishers, Oxford, UK, 1991). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was

isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and HindIII and subsequent agarose gel electrophoresis (0.8%).

The plasmid was called pEC-T18mob2 and is shown in Figure 1. It is deposited in the form of the strain Escherichia coli K-12 strain DH5αpEC-T18mob2 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 13244.

1.2 Preparation of the plasmid pEC-T18mob2zwf
The gene from Corynebacterium glutamicum ATCC13032 was first amplified by a polymerase chain reaction (PCR) by means of the following oligonucleotide primer:

zwf-forward:
5'-TCG ACG CGG TTC TGG AGC AG-3'

zwf-reverse:
5'-CTA AAT TAT GGC CTG CGC CAG-3'

The PCR reaction was carried out in 30 cycles in the presence of 200 µM deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), in each case 1 µM of the corresponding oligonucleotide, 100 ng chromosomal DNA from Corynebacterium glutamicum ATCC13032, 1/10 volume 10-fold reaction buffer and 2.6 units of a heat-stable Taq-/Pwo-DNA polymerase mixture (Expand High Fidelity PCR System from Roche Diagnostics, Mannheim, Germany) in a Thermocycler (PTC-100, MJ Research, Inc., Watertown, USA) under the following conditions: 94°C for 30 seconds, 64°C for 1 minute and 68°C for 3 minutes.

The amplified fragment about 1.8 kb in size was subsequently ligated with the aid of the SureClone Ligation Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) into the SmaI cleavage site of the vector pUC18 in accordance with

the manufacturer's instructions. The E. coli strain DH5α mcr (Grant et al., Proceedings of the National Academy of Sciences of the United States of America USA (1990) 87: 4645-4649) was transformed with the entire ligation batch. Transformants were identified with the aid of their carbenicillin resistance on LB-agar plates containing 50 µg/mL carbenicillin. The plasmids were prepared from 7 of the transformants and checked for the presence of the 1.8 kb PCR fragment as an insert by restriction analysis. The recombinant plasmid formed in this way is called pUC18zwf in the following.

For construction of pEC-T18mob2zwf, pUC18zwf was digested with KpnI and SalI, and the product was isolated with the aid of the NucleoSpin Extraction Kit from Macherey-Nagel (Düren, Germany) in accordance with the manufacturer's instructions and then ligated with the vector pEC-T18mob2, which had also been cleaved with KpnI and SalI and dephosphorylated. The E. coli strain DH5αmcr (Grant et al., Proceedings of the National Academy of Sciences of the United States of America USA (1990) 87: 4645-4649) was transformed with the entire ligation batch. Transformants were identified with the aid of their tetracycline resistance on LB-agar plates containing 5 µg/mL tetracycline. The plasmids were prepared from 12 of the transformants and checked for the presence of the 1.8 kb PCR fragment as an insert by restriction analysis. One of the recombinant plasmids isolated in this manner was called pEC-T18mob2zwf (Figure 2).

Example 2

Preparation of amino acid producers with an amplified zwf gene

The L-lysine-producing strain Corynebacterium glutamicum DSM5715 is described in EP-B-0435132 and the L-threonine-producing strain Brevibacterium flavum DSM5399 is described

in EP-B-0385940. Both strains are deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures] in Braunschweig (Germany) in accordance with the Budapest Treaty.

2.1 Preparation of the strains DSM5715/pEC-T18mob2zwf and DSM5399/pEC-T18mob2zwf

The strains DSM5715 and DSM5399 were transformed with the plasmid pEC-T18mob2zwf using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)) Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915 -927), cleaved with the restriction endonucleases XbaI and KpnI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strains obtained in this way were called DSM5715/pEC-T18mob2zwf and DSM5399/pEC-T18mob2zwf.

2.2 Preparation of L-threonine

The C. glutamicum strain DSM5399/pEC-T18mob2zwf obtained in Example 2.1 was cultured in a nutrient medium suitable for the production of threonine and the threonine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml

medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of threonine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD	L-Threonin g/l
DSM5399	12.3	0.74
DSM5399/pEC-T18mob2zwf	10.2	1.0

2.3 Preparation of L-lysine

The C. glutamicum strain DSM5715/pEC-T18mob2zwf obtained in Example 2.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l

Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	58 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 2.

Table 2

Strain	OD	L-Lysine HCl g/l
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	7.2	17.1

Example 3

Construction of a gene library of Corynebacterium glutamicum strain AS019

A DNA library of Corynebacterium glutamicum strain AS019 (Yoshihama et al., Journal of Bacteriology 162, 591-597 (1985)) was constructed using λ Zap Express™ system, (Short et al., (1988) Nucleic Acids Research, 16: 7583-7600), as described by O'Donohue (O'Donohue, M. (1997). The

Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from Corynebacterium glutamicum. Ph.D. Thesis, National University of Ireland, Galway). λ Zap Express™ kit was purchased from Stratagene (Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037) and used according to the manufacturers instructions. AS019-DNA was digested with restriction enzyme Sau3A and ligated to BamHI treated and dephosphorylated λ Zap Express™ arms.

Example 4

Cloning and sequencing of the pgi gene

1. Cloning

Escherichia coli strain DF1311, carrying mutations in the pgi and pgl genes as described by Kupor and Fraenkel, (Journal of Bacteriology 100: 1296-1301 (1969)), was transformed with approx. 500 ng of the AS019 λ Zap Express™ plasmid library described in Example 3. Selection for transformants was made on M9 minimal media, (Sambrook et al., (1989). Molecular Cloning. A Laboratory Manual Cold Spring Harbour Laboratories, USA), containing kanamycin at a concentration of 50 mg/l and incubation at 37°C for 48 hours. Plasmid DNA was isolated from one transformant according to Birnboim and Doly (Nucleic Acids Research 7: 1513-1523 (1979)) and designated pAMC1 (Figure 3).

2. Sequencing

For sequence analysis of the cloned insert of pAMC1 the method of Sanger et al. (Proceedings of the National Academy of Sciences USA 74,5463-5467 (1977)) was applied using primers differentially labelled with a coloured fluorescent tag. It was carried out using the ABI prism 310 genetic analyzer from Perkin Elmer Applied Biosystems, (Perkin Elmer Corporation, Norwalk, Connecticut, U.S.A), and the ABI prism Big Dye™ Terminator Cycle Sequencing Ready Reaction kit also from Perkin Elmer.

Initial sequence analysis was carried out using the universal forward and M13 reverse primers obtained from Pharmacia Biotech (St. Albans, Herts, AL1 3AW, UK):

Universal forward primer: GTA ATA CGA CTC ACT ATA GGG C

M13 reverse primer: GGA AAC AGC TAT GAC CAT G

Internal primers were subsequently designed from the sequence obtained which allowed the entire pgi gene to be deduced. The sequence of the internal primers is as follows:

Internal primer 1: GGA AAC AGG GGA GCC GTC

Internal primer 2: TGC TGA GAT ACC AGC GGT

Sequence obtained was then analyzed using the DNA Strider programme, (Marck, (1988). Nucleic Acids Research 16: 1829-1836), version 1.0 on an Apple Macintosh computer. This program allowed for analyses such as restriction site usage, open reading frame analysis and codon usage determination. Searches between DNA sequence obtained and those in EMBL and Genbank databases were achieved using the BLAST programme, (Altschul et al., (1997). Nucleic Acids Research, 25: 3389-3402). DNA and protein sequences were aligned using the Clustal V and Clustal W programs (Higgins and Sharp, 1988 Gene 73: 237-244).

The sequence thus obtained is shown in SEQ ID NO 1. The analysis of the nucleotide sequence obtained revealed an open reading frame of 1650 base pairs which was designated as pgi gene. It codes for a protein of 550 amino acids shown in SEQ ID NO 2.

Example 5

Preparation of an integration vector for integration mutagenesis of the pgi gene

An internal segment of the pgi gene was amplified by polymerase chain reaction (PCR) using genomic DNA isolated from Corynebacterium glutamicum AS019, (Heery and Dunican,

(1993) Applied and Environmental Microbiology 59: 791-799), as template. The pgi primers used were:

fwd. Primer: ATG GAR WCC AAY GGH AA
rev. Primer: YTC CAC GCC CCA YTG RTC
with R=A+G; Y=C+T; W=A+T; H=A+T+C.

PCR Parameters were as follows: 35 cycles
94°C for 1 min.
47°C for 1 min.
72°C for 30 sec.
1.5 mM $MgCl_2$
approx. 150-200 ng DNA template.

The PCR product obtained was cloned into the commercially available pGEM-T vector received from Promega Corp., (Promega UK, Southampton.) using strain E. coli JM109, (Yanisch-Perron et al., 1985. Gene, 33: 103-119), as a host. The sequence of the PCR product is shown as SEQ ID NO 3. The cloned insert was then excised as an EcoRI fragment and ligated to plasmid pBGS8 (Spratt et al., Gene 41: 337-342 (1986)) pretreated with EcoRI. The restriction enzymes used were obtained from Boehringer Mannheim UK Ltd., (Bell Lane, Lewes East Sussex BN7 1LG, UK.) and used according to manufacturers instructions. E. coli JM109 was then transformed with this ligation mixture and electrotransformants were selected on Luria agar supplemented with IPTG (isopropyl-ß-D-thiogalactopyranoside), XGAL (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and kanamycin at a concentration of 1 mM, 0.02% and 50 mg/l respectively. Agar plates were incubated for twelve hours at 37°C. Plasmid DNA was isolated from one transformant, characterized by restriction enzyme analysis using EcoRI, BamHI and SalI designated pMC1 (Figure 4).

Plasmid pMC1 was deposited in the form of Escherichia coli strain DH5a/pMC1 at the Deutsche Sammlung für

Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as DSM 12969 according to the Budapest treaty.

Example 6

Integration mutagenesis of the pgi gene in the lysine producer DSM 5715

The vector pMC1 mentioned in Example 5 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an AEC-resistant lysine producer. The vector pMC1 cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pMC1 integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin. For detection of the integration, the internal pgi fragment (Example 5) was labelled with the Dig hybridization kit from Boehringer Mannheim by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a transformant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybrization kit from Boehringer. It was found in this way that the plasmid pMC1 was inserted within the chromosomal pgi gene of strain DSM5715. The strain was called DSM5715::pMC1.

Example 7

Effect of over-expression of the zwf gene with simultaneous elimination of the pgi gene on the preparation of lysine

7.1 Preparation of the strain DSM5715::pMC1/pEC-T18mob2zwf

The vector pEC-T18mob2zwf mentioned in Example 1.2 was electroporated by the electroporation method of Tauch et al. (1994, FEMS Microbiological Letters, 123:343-347) in Corynebacterium glutamicum DSM 5715::pMC1. Selection for plasmid-carrying cells was made by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 15 mg/l kanamycin and with 5 mg/l tetracycline. Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by treatment with the restriction enzymes KpnI and SalI with subsequent agarose gel electrophoresis. The strain was called DSM5715::pMC1/pEC-T18mob2zwf.

7.2 Preparation of lysine

The C. glutamicum strain DSM5715::pMC1/pEC-T18mob2zwf obtained in Example 7.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours at 33°C. The cultures of the comparison strains were supplemented according to their resistance to antibiotics. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

Strain	OD	L-Lysine HCl g/l
DSM5715	7.3	14.3
DSM5715/pEC-T18mob2zwf	7.1	14.6
DSM5715::pMC1/ pECTmob2zwf	10.4	15.2

Example 8

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al., (1995, Plasmid 33:168-179), and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vektor Kit, Code no. 251301) was cleaved

with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 9

Isolation and sequencing of the poxB gene

The cosmid DNA of an individual colony (Example 7) was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the

cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 µg/ml zeocin. The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems(Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the

pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 4. Analysis of the nucleotide sequence showed an open reading frame of 1737 base pairs, which was called the poxB gene. The poxB gene codes for a polypeptide of 579 amino acids (SEQ ID NO. 5).

Example 10

Preparation of an integration vector for integration mutagenesis of the poxB gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the poxB gene known for C. glutamicum from Example 8, the following oligonucleotides were chosen for the polymerase chain reaction:

poxBint1:
5` TGC GAG ATG GTG AAT GGT GG 3`
poxBint2:
5` GCA TGA GGC AAC GCA TTA GC 3`

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the polymerase chain reaction, a DNA fragment approx. 0.9 kb in

size was isolated, this carrying an internal fragment of the poxB gene and being shown in SEQ ID No. 6.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead at al. (1991) Bio/Technology 9:657-663). The E. coli strain DH5α was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol.I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1poxBint (Figure 5).

Plasmid pCR2.1poxBint has been deposited in the form of the strain Escherichia coli DH5α/pCR2.1poxBint as DSM 13114 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 11

Integration mutagenesis of the poxB gene in the lysine producer DSM 5715

The vector pCR2.1poxBint mentioned in Example 10 was electroporated by the electroporation method of Tauch et al.(FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1poxBint

cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pCR2.1poxBint integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin. For detection of the integration, the poxBint fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybrization kit from Boehringer. The plasmid pCR2.1poxBint mentioned in Example 9 had been inserted into the chromosome of DSM5715 within the chromosomal poxB gene. The strain was called DSM5715::pCR2.1poxBint.

Example 12

Effect of over-expression of the zwf gene with simultaneous elimination of the poxB gene on the preparation of lysine

12.1 Preparation of the strain DSM5715::pCR2.1poxBint/pEC-T18mob2zwf

The strain DSM5715::pCR2.1poxBint was transformed with the plasmid pEC-T18mob2zwf using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-

agar, which had been supplemented with 5 mg/l tetracycline and 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927), cleaved with the restriction endonucleases XbaI and KpnI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained in this way was called DSM5715:pCR2.1poxBint/pEC-T18mob2zwf.

12.2 Preparation of L-lysine

The C. glutamicum strain DSM5715::pCR2.1poxBint/pEC-T18mob2zwf obtained in Example 12.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours at 33°C.The comparison strains were supplemented according to their resistance to antibiotics. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	58 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 4.

Table 4

Strain	OD	L-Lysine HCl g/l
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	8.3	17.1
DSM5715::pCR2.1poxBint	7.1	16.7
DSM5715::pCR2.1poxBint/ pEC-Tmob2zwf	7.8	17.7

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0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.90 (updated 08.03.2000)
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1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	10
1-2	line	24-29
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	20 January 2000 (20.01.2000)
1-3-4	Accession Number	DSMZ 13244
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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Patent claims

1. A process for the preparation of L-amino acids by fermentation of coryneform bacteria
which comprises
carrying out the following steps:
a) fermentation of the desired L-amino acid-producing bacteria in which at least the zwf gene is amplified,
b) concentration of the L-amino acid in the medium or in the cells of the bacteria and
c) isolation of the L-amino acid produced.

2. The process as claimed in claim 1,
wherein
bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally amplified, in particular over-expressed, are employed.

3. The process as claimed in claim 1,
wherein
coryneform bacteria which prepare L-threonine, L-lysine or L-tryptophan are used.

4. The process as claimed in claim 3,
wherein
coryneform bacteria which prepare L-lysine are used.

5. A process for the fermentative preparation of L-lysine as claimed in claim 2,
wherein
in the coryneform microorganisms which in particular already produce L-lysine, one or more genes chosen from the group consisting of

5.1 the dapA gene which codes for dihydrodipicolinate synthase,

5.2 the lysC gene which codes for a feed back resistant aspartate kinase,

5.3 the gap gene which codes for glyceralaldehyde 3-phosphate dehydrogenase,

5.4 the pyc gene which codes for pyruvate carboxylase,

5.5 the tkt gene which codes for transketolase,

5.6 the gnd gene which codes for glucose 6-phosphate dehydrogenase,

5.7 the lysE gene which codes for lysine export,

5.8 the zwa1 gene,

5.9 the eno gene which codes for enolase

is or are amplified or over-expressed at the same time.

6. A process for the fermentative preparation of L-threonine as claimed in claim 2,
w h e r e i n
in the coryneform microorganisms which in particular already produce L-threonine, one or more genes chosen from the group consisting of

6.1 the hom gene which codes for homoserine dehydrogenase or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase,

6.2 the gap gene which codes for glyceralaldehyde 3-phosphate dehydrogenase,

6.3 the pyc gene which codes for pyruvate carboxylase,

6.4 the mqo gene which codes for malate:quinone oxidoreductase,

6.5 the tkt gene which codes for transketolase,

6.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,

6.7 the thrE gene which codes for threonine export,

6.8 the zwa1 gene,

6.9 the eno gene which codes for enolase

is or are amplified, in particular over-expressed, at the same time.

7. The process as claimed in claim 2,
w h e r e i n
for the preparation of L-amino acids, in particular L-lysine or L-threonine, bacteria in which one or more genes chosen from the group consisting of,

7.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,

7.2 the pgi gene which codes for glucose 6-phosphate isomerase

7.3 the poxB gene which codes for pyruvate oxidase or

7.4 the zwa2 gene

is or are attenuated at the same time, are fermented.

8. The process as claimed in claims 2 to 6,
w h e r e i n
to achieve the amplification, the number of copies of

the genes or nucleotide sequences is increased by transformation of the microorganisms with plasmid vectors which carry these genes or nucleotide sequences.

9. The plasmid vector pEC-T18mob2 deposited under the designation DSM13244 in E.coli K-12 DH5α, shown in Figure 2.

10. A coryneform microorganism, in particular of the genus Corynebacterium, transformed by the introduction of the plasmid vector as claimed in claim 9, which additionally contains the zwf gene.

Figure 1: Map of the plasmid pEC-T18mob2

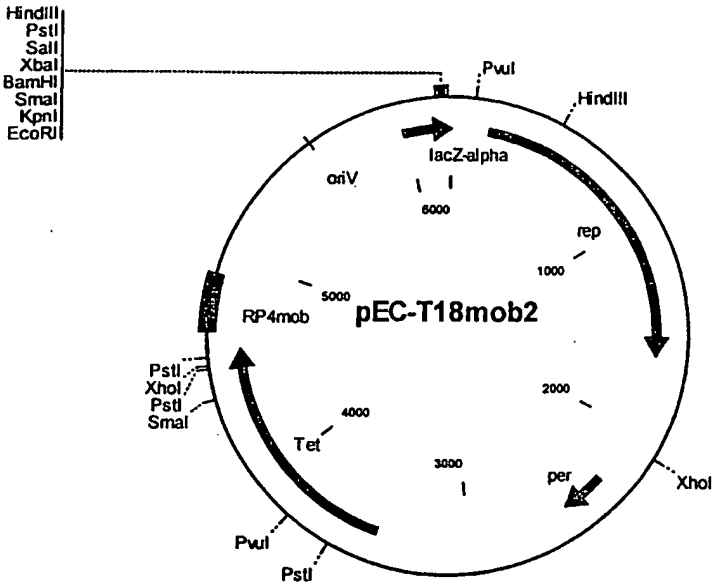


Figure 2: Map of the plasmid pEC-T18mob2zwf

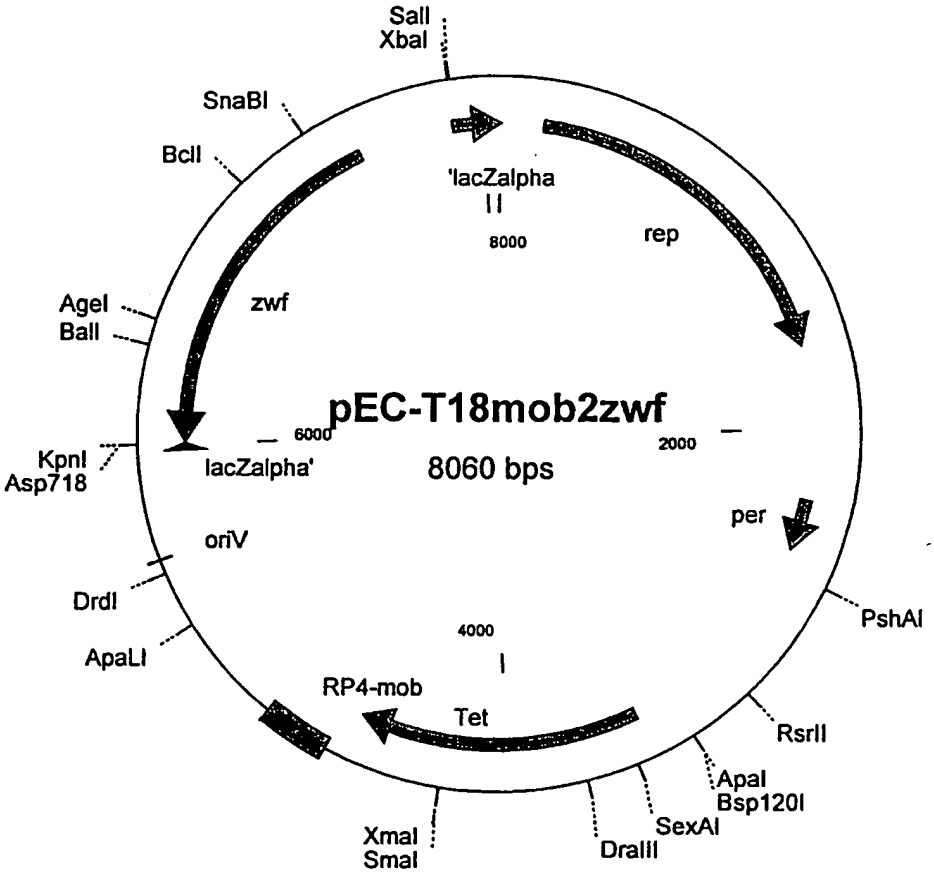
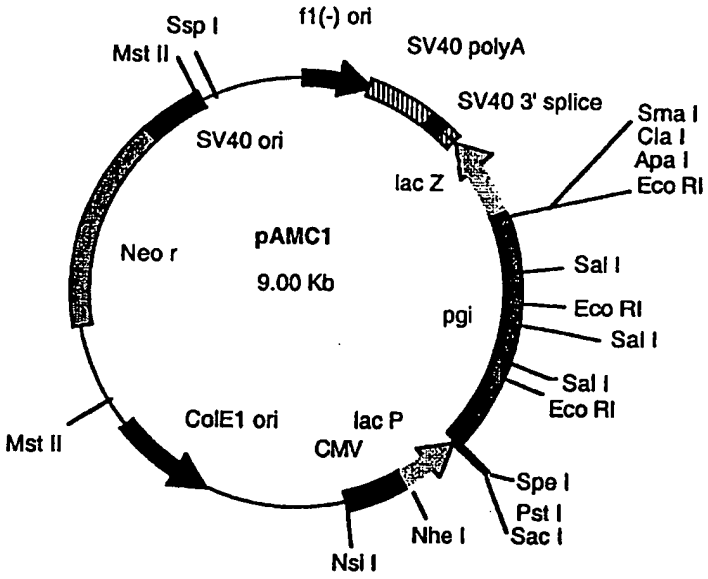


Figure 3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9.00 Kb
Neo r
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Sal I
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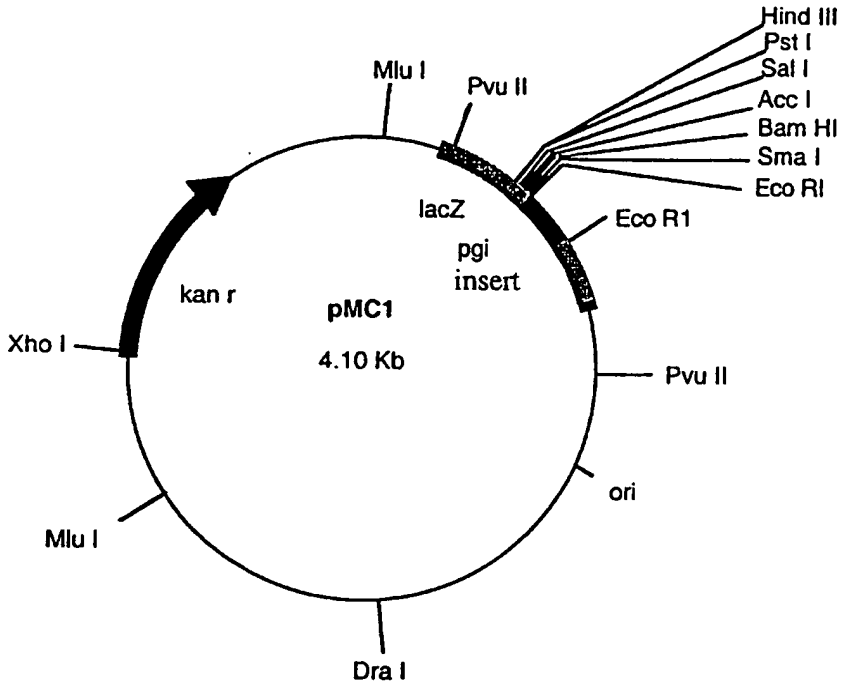
Figure 4: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Sal I
Acc I
Bam HI
Sma I
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Mlu I
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kan r
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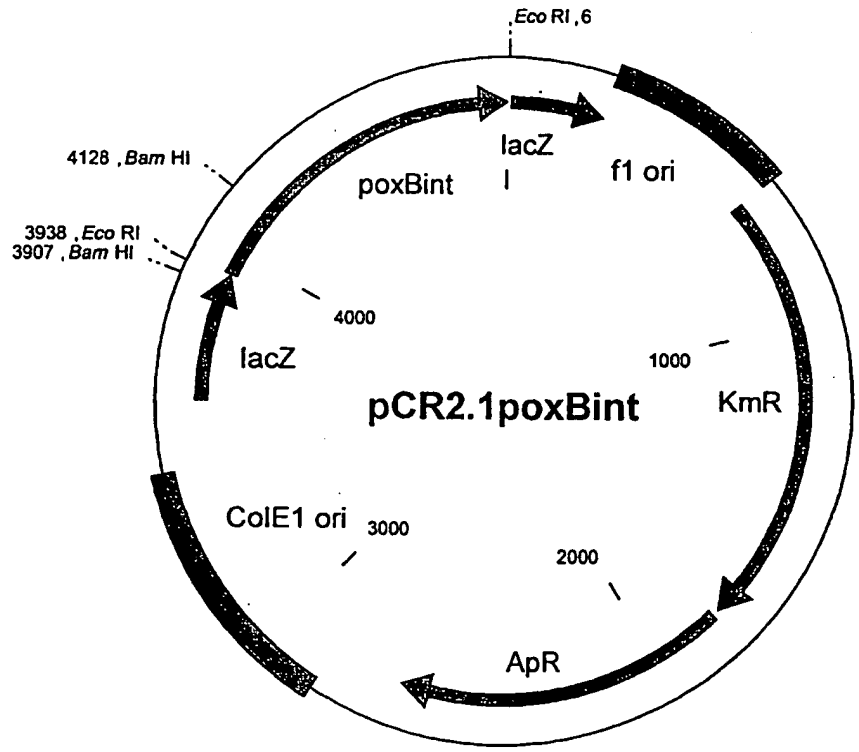
4.10 Kb
Xho I
Pvu II
ori
Mlu I
Dra I

Figure 5: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f1 ori
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poxBint
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SEQUENCE PROTOCOL

<110> Degussa-Hüls AG
National University of Ireland, Galway
Forschungszentrum Jülich GmbH

<120> Process for the fermentative preparation of L-amino acids using the zwf gene.

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Pro Ala Glu Ala Asp Leu Ser Val Asp Gly Gln Asp Val Ala Ala Asp
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Val His Glu Val Leu Gly Arg Met Arg Asp Phe Ala Thr Ala Leu Arg
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Ser Gly Asn Trp Leu Gly His Thr Gly His Thr Ile Lys Lys Ile Val
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Tyr Gly Ala Glu Thr His Ala Val Leu Pro Tyr Ser Glu Asp Leu Ser
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465                 470                 475                 480

Pro Ser Ile Leu Gly Ala Leu Ile Ala Leu Tyr Glu His Thr Val Met
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 90                  95                 100                 105

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Leu Val Glu Gly Gln Pro Glu Phe Gly Thr Asp His Glu Glu Val Asn
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ttc gca gag att gcg gcg gct gcg ggt atc aaa tcg gta cgc atc acc   1841
Phe Ala Glu Ile Ala Ala Ala Ala Gly Ile Lys Ser Val Arg Ile Thr
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gat ccg aag aaa gtt cgc gag cag cta gct gag gca ttg gca tat cct   1889
Asp Pro Lys Lys Val Arg Glu Gln Leu Ala Glu Ala Leu Ala Tyr Pro
                510                 515                 520

gga cct gta ctg atc gat atc gtc acg gat cct aat gcg ctg tcg atc   1937
Gly Pro Val Leu Ile Asp Ile Val Thr Asp Pro Asn Ala Leu Ser Ile
            525                 530                 535

cca cca acc atc acg tgg gaa cag gtc atg gga ttc agc aag gcg gcc   1985
Pro Pro Thr Ile Thr Trp Glu Gln Val Met Gly Phe Ser Lys Ala Ala
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acc cga acc gtc ttt ggt gga gga gta gga gcg atg atc gat ctg gcc   2033
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cgt tcg aac ata agg aat att cct act cca tgatgattga tacacctgct     2083
Arg Ser Asn Ile Arg Asn Ile Pro Thr Pro
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Val Asp Ala Val Arg Gln Ser Asp Ile Glu Trp Val His Val Arg Asn
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Gln Ser Thr Met Ala Gly Lys Gly Val Ser Val Val Val Ile Pro Gly
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Ile Ser Ser Gly Thr Pro Val Val Phe Pro Asp Pro Thr Glu Ala Ala
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Ala Leu Val Glu Ala Ile Asn Asn Ala Lys Ser Val Thr Leu Phe Cys
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Gly Ala Gly Val Lys Asn Ala Arg Ala Gln Val Leu Glu Leu Ala Glu
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Lys Ile Lys Ser Pro Ile Gly His Ala Leu Gly Gly Lys Gln Tyr Ile
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Gln His Glu Asn Pro Phe Glu Val Gly Met Ser Gly Leu Leu Gly Tyr
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260 265 270

Gly Thr Asp Phe Pro Tyr Ser Asp Phe Leu Pro Lys Asp Asn Val Ala
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Gln Val Asp Ile Asn Gly Ala His Ile Gly Arg Arg Thr Thr Val Lys
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Tyr Pro Val Thr Gly Asp Val Ala Ala Thr Ile Glu Asn Ile Leu Pro
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His Val Lys Glu Lys Thr Asp Arg Ser Phe Leu Asp Arg Met Leu Lys
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Ala His Glu Arg Lys Leu Ser Ser Val Val Glu Thr Tyr Thr His Asn
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Val Glu Lys His Val Pro Ile His Pro Glu Tyr Val Ala Ser Ile Leu
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Asn Glu Leu Ala Asp Lys Asp Ala Val Phe Thr Val Asp Thr Gly Met
370 375 380

Cys Asn Val Trp His Ala Arg Tyr Ile Glu Asn Pro Glu Gly Thr Arg
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Asp Phe Val Gly Ser Phe Arg His Gly Thr Met Ala Asn Ala Leu Pro
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His Ala Ile Gly Ala Gln Ser Val Asp Arg Asn Arg Gln Val Ile Ala
420 425 430

Met Cys Gly Asp Gly Gly Leu Gly Met Leu Leu Gly Glu Leu Leu Thr
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Ser Leu Gly Met Val Lys Leu Glu Met Leu Val Glu Gly Gln Pro Glu
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Ala Gly Ile Lys Ser Val Arg Ile Thr Asp Pro Lys Lys Val Arg Glu
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Gln Leu Ala Glu Ala Leu Ala Tyr Pro Gly Pro Val Leu Ile Asp Ile
515 520 525

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gatcctactg aggctgcagc gctggtggag gcgattaaca acgctaagtc tgtcactttg 240
ttctgcggtg cgggcgtgaa gaatgctcgc gcgcaggtgt tggagttggc ggagaagatt 300
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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 00/06303

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N15/77 C12P13/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 09 224661 A (MITSUBISHI CHEM CORP) 2 September 1997 (1997-09-02) cited in the application the whole document & DATABASE WPI Section Ch, Week 199745 Derwent Publications Ltd., London, GB; Class B04, AN 1997-484096 HATAKEYAMA KAZUHISA ET AL.: "Glucose-6-phosphate dehydrogenase " & JP 09 224661 A (MITSUBISHI CHEM CORP), 2 September 1997 (1997-09-02) abstract --- -/--	9,10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 February 2001	21/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/06303

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L. EGGELING ET AL.: "L-Glutamate and L-Lysine: traditional products with impetuous developments" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 52, August 1999 (1999-08), pages 146-153, XP000979507 the whole document	1-10

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/EP 00/06303

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 9224661 A	02-09-1997	NONE	